

METHOD FOR DETECTING EUBACTERIA IN BIOLOGICAL SAMPLES WITH CATALYTICALLY INACTIVE MUREIN BINDING ENZYMES

FIELD OF THE INVENTION

The invention generally relates to microbiology and reagents and methods for identifying fungi and bacteria in samples of biological fluids, foods, water, air, solutions and the like; and, specifically, to diagnostic reagents and methods for detecting and quantifying fungi and bacteria, as well as, for distinguishing between bacteria and fungi in a sample.

BACKGROUND OF THE INVENTION

Rapid tests for identification of pathogenic bacteria, fungi and their products are becoming increasingly important to health care professionals, as well as individuals responsible for water and food safety. Timely identification and generic classification of etiologic agents is a key to preventing spread of disease, and is also effective to expedite patient treatment and thereby reduce costs associated with disease management.

Microbiological stains are diagnostic reagents capable of identifying bacteria and fungi from cultures, but they commonly are not useful as rapid diagnostic tests with patient samples because of cross-reactivity with natural mammalian and plant products and with other non-pathogenic microorganisms. While gram-staining for bacteria remains a most useful diagnostic criteria in evaluation of isolated bacterial cultures, the reagents and methods generally lacks sensitivity because many groups of bacteria stain either poorly or not at all. Similarly, histological staining of polysaccharides using Grocott methenamine silver nitrate as a test for fungi lacks sensitivity and is subject to confusing non-specific cross-reactions with e.g. connective tissue proteoglycans, glycosaminoglycans and mucins. Commonly, definitive identification and confirmation of infection, or contamination, requires multiple tedious steps of culture and multiple different testing methods and often these procedures are not suitable for use in smaller clinical test laboratories.

Bacterial envelopes are composed of an inner phospholipid bilayer with membrane proteins surrounded by a rigid shell of covalently linked peptidoglycan that is reactive with crystal violet and iodine, i.e., the envelope in gram positive bacteria. Gram-negative bacteria have the latter structure, but with lesser amounts of peptidoglycan, and their envelope includes an additional outer membrane characterized by lipopolysaccharide (LPS), porins and transport proteins. Peptidoglycan structures vary in different bacteria. In *E. coli* peptidoglycan, N-acetylglucosamine alternates with N-acetylmuramic acid in $\beta(1,4)$ -glycosidic bonds to form complex polymers with tetrapeptide side chains. The latter side chains are composed of L-Ala-D-Glu-mesodiaminopimelic acid-D-Ala. Cross-linking between peptidoglycan chains forms a gel that varies in consistency dependent upon the degree of cross-linking.

In contrast, most pathogenic fungi contain chitin in their cell walls, septa and spores, both in hyphal and yeast forms. Chitin is a $\beta 1 \rightarrow 4$ linked polymer of only 2-deoxy-2-acetamindoglucose (N-acetyl glucosamine, abbreviated GlcNAc). Chitin is also found in tissues of insects and crustaceans. Certain classes of fungi have cell walls that contain both chitins and murein-like compounds. However, the murein-like compounds are commonly hidden within

thick protective cell wall structures, and/or expressed only in low levels. Fungi, as eukaryotes, also have many similarities with mammalian cells, often making it more difficult to distinguish between patient cellular materials and fungal products.

Immunoassays used in clinical microbiology commonly rely on antibodies that, while highly specific, are also narrowly reactive, e.g., with a single defined epitope in a complex carbohydrate structure of a particular serotype of bacteria. Cross-reactivity of reagents with more than one different type of bacteria is often viewed as an undesirable performance attribute. Antibody reagents also frequently are unable to distinguish between a whole bacteria and the degradative products of a bacteria, and as a result breakdown products can act as 'confounding', or 'interfering, substances' in diagnostic assays. Where enzymes are used in immunoassays, they are commonly used to generate a detectable signal. For example, enzyme-linked immunosorbent assays (ELISA) such as those disclosed in U.S. Pat. Nos. 4,233,402 and 4,486,530, involve labeling an antibody (or antigen) by covalently linking it to a catalytically active enzyme. The presence (or amount) of the labeled compound may be determined in an assay by adding an enzyme substrate that produces a measurable signal (e.g., a colored product or fluorescence.) While certain assay formats rely upon re-activation of an inactivated-enzyme (e.g., U.S. Pat. No. 4,043,872), generation of a signal in an ELISA commonly requires a catalytically active enzyme, and preferably one having giving a rapid production of product (i.e., a high turnover number.) Catalytically active enzymes considered for possible use in diagnostic immunoassays include those hydrolyzing glycosidic bonds (e.g., U.S. patent Ser. Nos. 4,208,479 at column 17; 4,299,916 at column 33.)

Enzymes are given names indicating both the principal substrate and the reaction catalyzed. However, few enzymes are absolutely specific to the structure of a particular substrate and most can act on closely related structural analogues of their physiological substrates, although usually at reduced rates. The Commission on Enzymes of the International Union of Biochemistry has evolved a systematic nomenclature for enzymes based on the reactions catalyzed. Lysozyme, classified as a glycoside hydrolase in IUB class EC3.2.1 (i.e., IUB class EC3.2.1.17), has specificity for compounds containing N-acetylmuramic acid and a peptide side chain (i.e., mureins.) Lysozymes from different species catalyze hydrolysis of $\beta(1,4)$ bonds between N-acetylmuramic acid and adjacent sugar residues in mureins and chitins, but chitins more slowly. Chitinase, classified as an N-glycosyl hydrolase (i.e., IUB class EC3.2.1.14), binds and degrades chitin and murein, but murein much more slowly than chitin.

It would be highly desirable for clinical test laboratories to have access to reagents that, while specifically reactive with many genera of bacteria and fungi, are also useful as reagents in assays that distinguish between bacterial and fungal infection or contamination. However, the array of different antigens in bacteria and fungi that are available as potential targets for development of immunoassays is somewhat bewildering. Also, increasingly laboratory personnel are being placed at a potential risk of exposure to debilitating or life threatening diseases by contact with infected patient samples. While it is commonly an aim to conduct all assays with non-infectious materials, the resultant fixed and killed bacterial samples often contain denatured antigens that are poorly reactive with assay reagents. Diagnostic reagents reactive with fixed, and/or killed and dead bacterial and fungal products are highly desirable.